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STUDIES ON A Ca^{2+}-DEPENDENT ATPase OF HUMAN ERYTHROCYTE MEMBRANES

EFFECTS OF Ca^{2+} AND H^+

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SUMMARY

The kinetics of the Ca^{2+} activation of human erythrocyte membrane Ca^{2+}-dependent ATPase (ATP-phosphohydrolase, EC 3.6.1.3) was investigated between pH 5.8 and 8.6.

From the log $\beta\tilde{V}^{H^+}$–pH and $p\tilde{K}_m{}^{H^+}$–pH plots it can be concluded that MgATP is the substrate of this ATPase. HATP and HMgATP do not act as substrates. The K_m value at pH 7.0 is equal to 50 μM, and the apparent Ca^{2+} dissociation constant in the presence of 2 mM Mg^{2+} is 0.92 μM.

Ca^{2+} is an autosteric effector of the Ca^{2+}-ATPase. It is not essential for substrate binding, but for substrate splitting. In the absence of Ca^{2+}, an inactive enzyme substrate complex is formed. The K_m value of this complex does not differ significantly from the K_m value of the active enzyme–Ca^{2+}–substrate complex.

The catalytic centre of the examined Ca^{2+}-ATPase contains three different ionizable groups as follows:

1. One ionizable group with pK of about 5.8. This group is active when dissociated. It is involved in the process of substrate binding. Presumably it is identical with an imidazole nitrogen of histidine.

2. A second group with pK of about 5.8. This group is also active when dissociated. It is involved in the process of substrate splitting. It may also be an imidazole nitrogen of histidine.

3. One ionizable group with pK 8.2. This group is active when undissociated. It is involved in the process of substrate splitting and it may be identical with an α-amino group of any amino acid.

INTRODUCTION

Besides the well-known (Na^+,K^+) dependent ATPase of human erythrocyte membranes, related to the active transport of Na^+ and K^+ across the erythrocyte membrane by Post *et al.*[1], some authors described an ouabain-insensitive part of the erythrocyte ATPase system[1-5]. This part, possibly consisting of more than one enzyme, is activated by the simultaneous presence of Mg^{2+} and Ca^{2+} (refs 2–5).

Subsequent investigations on this ouabain-insensitive, (Mg^{2+}, Ca^{2+})-dependent ATPase system showed a number of quite interesting results. Wins and Schoffeniels[6] could demonstrate that the shrinkage of erythrocyte ghosts is linked to the presence of ATP and Ca^{2+} inside the ghosts. They hypothesized that the ghost contraction involves the activity of an actomyosin-like system in human red cells The existence of such a system was demonstrated by Ohnishi[7] in 1962. In addition, Rosenthal *et al.*[8] could isolate from human red cells a fibrillar protein, which exhibits Ca^{2+}-dependent ATPase activity.

A most interesting feature of this enzyme system is its relation to active outward transport of Ca^{2+}, as shown by Schatzmann and Vincenzi[9-14]. This result appears to be in contrast to the finding of Wins and Schoffeniels[6].

Recent studies, however, show that this enzyme system is not only activated by very low Ca^{2+} concentrations[12-13], but also, at least in part, by Na^+ or K^+ (refs 14 and 15). The conclusion that there might be more than one Ca^{2+}-dependent ATPase present in human erythrocyte membranes, is supported by the kinetic evidence of two enzymes, which show different K_m values[15].

The effects of Ca^{2+} and H^+ on the reaction rate of a Ca^{2+}-dependent ATPase of human erythrocyte membranes are reported in this paper, as part of a programme to characterize the Ca^{2+}-dependent ATPase system with respect to kinetic and enzymological properties.

MATERIALS AND METHODS

Chemicals

Oxidized glutathione was purchased from Serva, Heidelberg; ATP (as the disodium salt) from C. F. Boehringer und Soehne, Mannheim and E. Merck, Darmstadt; *trans*-cyclohexyldiamintetraacetate (Chel-CD) from Fluka AG, Switzerland; sucrose from Schuchardt, München; purified N_2 from Linde AG, Mainz–Kostheim, and all other chemicals from E. Merck, Darmstadt. In all cases the metal ions were used as chlorides. The water was twice redistilled in a quartz apparatus.

Preparation of the Ca^{2+}-dependent ATPase

The enzyme was prepared essentially according to the method described earlier[13] with the following changes:

The hemolysis and the washings were carried out in the presence of 20 and 2 mM sucrose, respectively. The glycine buffer present in the hemolysis solution was adjusted to pH 10.2. Thus, a pH value of 9.5 resulted after hemolysis. This value was lowered to pH 9.2 by adding 1 M Tris–HCl (pH approx. 7) after 1 min. At this step the Na^+ concentration was increased to 50 mM and maintained at this level during the steps of washing and freezing There was no indication of any change of kinetic constants after the exposure of the enzyme to pH 9.5.

The original enzyme preparation[13] yielded $1/v$ *versus* $1/[MgATP]$ plots, which were nonlinear at pH values > 7.6. Mathematical analysis of the curves led to the hypothesis that two Ca^{2+}-dependent ATPases with entirely different K_m values exist, *i.e.* a component with high substrate affinity (K_m = 10 μM at pH 8.8) and a component with low substrate affinity (K_m = 440 μM at pH 8.8; ref. 15). The enzyme with low substrate affinity might be identidal with the low Ca^{2+} affinity

component described by Schatzmann and Rossi[14]. As a result of the above mentioned modification of the original preparation procedure the activity of the enzyme with low substrate affinity was no longer detectable in the enzyme preparation: the $1/v$ *versus* $1/[MgATP]$ plots became linear all over the concentration range of MgATP (0.02–1.0 mM) at any pH-value between 5.8 and 8.6. The K_m-values of this preparation, which were obtained at various pH values, did not differ from those of the high substrate affinity enzyme present in the original preparation (ref. 15). The results obtained here indicate that the present preparation consists of an enzyme, which is uniform with respect to its kinetic properties.

In order to ensure that different batches of the enzyme preparation used had equal kinetic properties, each enzyme preparation under investigation was tested daily by determining its Ca^{2+} dissociation constant at pH 7.0. Changes in these values indicate a partial denaturation of the enzyme more sensitively than the K_m value, the Na^+ dissociation constant or the specific activity.

Determination of enzyme activity

The conditions for activity measurements are reported in Results and Discussion. The test volume was 10.0 ml throughout, the temperature 30.0 °C. The test medium contained 10 mM Tris–maleate buffer and 20 μg enzyme protein per ml.

Since the reaction rate of the enzyme is dependent on the Na^+ concentration (ref. 15), the experiments were carried out in the presence of an optimum concentration of 100 mM Na^+ throughout.

2.0-ml aliquots of the test solution were taken immediately after addition of substrate and after 5, 10, and 15 min and mixed with 2.0 ml of 0.125 M trichloroacetic acid. After centrifugation to remove the precipitated protein, P_i was determined by the method of Fiske and SubbaRow[16] as modified by Lacy[17], by using ascorbic acid as reductant. P_i determinations were carried out in a Technicon AutoAnalyzer, using the following concentrations of test reagents: [ammoniummolybdate], 40 g/l; [ascorbic acid], 2 g/l; washing solutions: according to the respective test solution. In order to prevent adsorption and desorption of P_i in the tube system of the AutoAnalyzer, 10 μM P_i was added to the ascorbic acid solution. Due to this P_i "background", the absorbance of the phosphomolybdate complex was exactly proportional to the concentration of the released P_i within the experimental range of the P_i concentration.

To prevent any undesirable effects due to an oxidation of SH groups by atmospheric oxygen and to maintain a constant redox potential of the reaction system, the tests were performed in the presence of 4 mM reduced and 0.2 mM oxidized glutathione.

Ca^{2+} showed a stimulating effect at such low concentrations that the application of Ca^{2+} buffers was necessary. The buffer system consisted of 0.4 mM EDTA and 0.4 mM (Mg^{2+} + Ca^{2+}). The value of $[Ca^{2+}]_t$, which was necessary to adjust the desired concentration of free Ca^{2+}, was calculated by using Eqn 11 (see Appendix) and the following dissociation constants (T = 25–30 °C, 100 mM NaCl or KCl): $K_{MgEDTA} = 2.1 \cdot 10^{-9}$ M, $K_{CaEDTA} = 3.8 \cdot 10^{-11}$ M (ref. 18) For further information see Appendix.

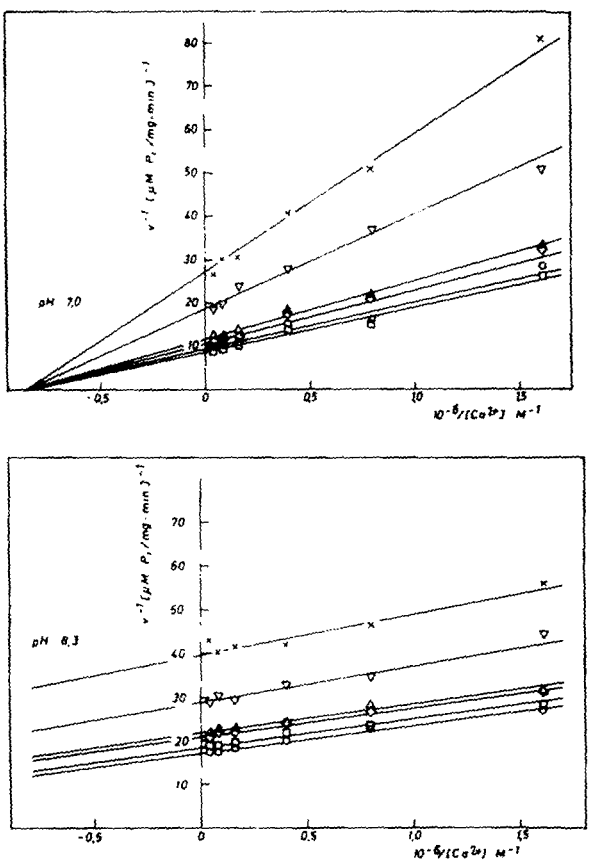
Care was taken to ensure that the turnover of ATP amounted to 5 % and never exceeded 10 % of the total ATP concentration. The reaction rate was linear in all cases within the first 15 min.

Measurements of the reaction rate at pH < 5.8 and pH > 8.8 were not possible, since the enzyme becomes unstable at these pH ranges. In addition, at pH values < 5.8 the Ca^{2+} buffer used in the experiments becomes ineffective due to protonization of EDTA (*cf.* Appendix), and the exposure of the enzyme preparation to pH values > 8.8 causes an alteration of the membrane proteins, which results in a severe disturbance of the P_i determination in the AutoAnalyzer.

RESULTS AND DISCUSSION

The reaction rate of the Ca^{2+}-dependent ATPase of human erythrocyte membranes was measured as a function of the Ca^{2+}, H^+ and substrate concentrations. The experiments were carried out within the range of pH 5.8–8.6 in steps of 0.3–0.5 pH unit.

When $1/v$ is plotted *versus* $1/[Ca^{2+}]$, straight lines appear all over the Ca^{2+}

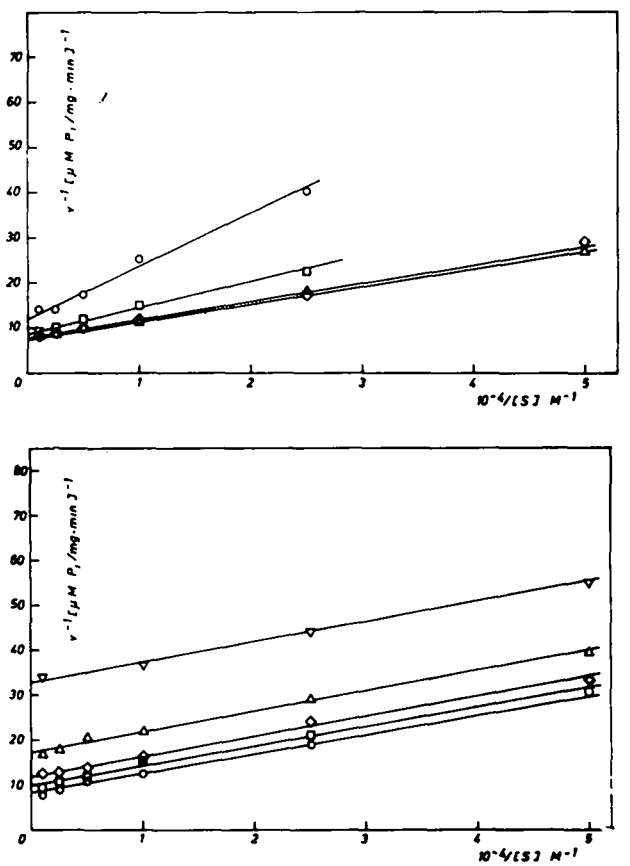


Figs 1 and 2. Two examples of the $1/v$–$1/[Ca^{2+}]$ plots of the Ca^{2+}-ATPase reaction rate at variable Ca^{2+}, H^+ and MgATP concentrations. pH as indicated in the figures. $[Na^+] = 100$ mM, $[Mg^{2+}] = 2$ mM. The Ca^{2+} concentration was adjusted by 0.4 mM Ca^{2+}–EDTA buffer as described in the appendix. [MgATP]: ○, 1 mM; □, 0.4 mM; ◇, 0.2 mM; △, 0.1 mM; ▽, 0.04 mM and ×, 0.02 mM. From all other experimental data only the intercepts of the straight lines ($1/v_{[Ca^{2+}]=\infty}$) are presented in Figs. 3 and 4.

concentration range at any pH value tested. At pH 5.8 there is one intersection point between all straight lines in the second quadrant of the coordinate system. Between pH 6.3 and 7.0 this intersection point appears at the negative part of the abscissa (Fig. 1). At pH values > 7.0 the intersection point between all straight lines disappears and the lines, especially those obtained at high substrate concentrations, become parallel (Fig. 2).

Extrapolation of the straight lines yields the limiting values of $1/v$ at $[Ca^{2+}] = \infty$. Replotting of these values *versus* the reciprocal MgATP concentration (Figs 3 and 4) leads to the values of $1/\beta\tilde{V}^{H^+}$ and $\tilde{K}_m{}^{H^+}$ at $[Ca^{2+}] = \infty$, which are used to perform the log $\beta\tilde{V}^{H^+}$–pH and p$\tilde{K}_m{}^{H^+}$–pH plots (Fig. 5).

From the log $\beta\tilde{V}^{H^+}$–pH and the p$\tilde{K}_m{}^{H^+}$–pH plots the pK values of all ionizable groups of the enzyme and substrate molecules are obtained, which are involved in binding and splitting the substrate. In the log $\beta\tilde{V}^{H^+}$–pH plot of Fig. 5



Figs 3 and 4. Plot of the intercept of the straight lines obtained from $1/v$–$1/[Ca^{2+}]$ plots *versus* the reciprocal MgATP concentration. From the resulting straight lines, the $\beta\tilde{V}^{H+}$ and $\tilde{K}_m{}^{H+}$ values required in the log $\beta\tilde{V}^{H+}$–pH and p$\tilde{K}_m{}^{H+}$–pH plots (Fig. 5) are evaluated. pH values: Fig. 3: ○, pH 5.8; □, pH 6.3; ◇, pH 6.6; △, pH 7.0; Fig. 4: ○, pH 7.3; □, pH 7.6; ◇, pH 7.9; △, pH 8.3; ▽, pH 8.6.

two inflection points of the curve appear at pH 8.2 and at about 5.8, which can be referred to two ionizable groups of the enzyme–substrate complex. These functional groups are essential for the cleavage of the substrate. The shape of the curve indicates that the enzyme–substrate complex is active in the range between the two

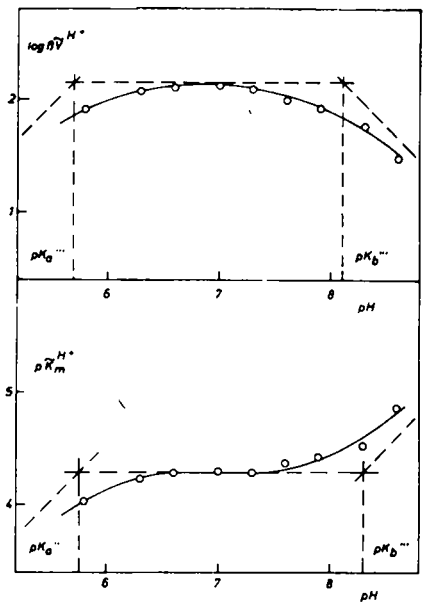


Fig. 5. $\log\beta\tilde{V}^{H+}$–pH and $p\tilde{K}_m{}^{H+}$–pH plots at $[Ca^{2+}] = \infty$ for the evaluation of the ionizable groups of free substrate, free enzyme and the enzyme–substrate complex. Values are obtained from Figs. 3 and 4. The curves are drawn considering that they miss the intersection point of the extrapolated neighbouring straight parts by a vertical distance of log 2.

inflection points, whereas it is inactive in the stage of ionization, which becomes predominant above pH 8.2. In addition, it may be suggested that the enzyme–substrate complex is also inactive below a pH value of about 5.8, although this is uncertain in some degree because of the experimental difficulties mentioned above.

As expected, the $p\tilde{K}_m{}^{H+}$–pH plot shows an inflection, which corresponds to the inflection point of the $\log\beta\tilde{V}^{H+}$–pH plot at pH 8.2. This means that the functional group of the enzyme–substrate complex with pK 8.2, which can be derived from the $\log\beta\tilde{V}^{H+}$–pH plot, has no essential function in binding the substrate, if at all present in the free enzyme.

In contrast to the shape of the curves at pH 8.2, the inflection of the $p\tilde{K}_m{}^{H+}$–pH curve proceeds at pH 5.8 into the opposite direction as expected. This phenomenon indicates the existence of two functional groups with pK values of about 5.8 at the free enzyme or the free substrate, in addition to the functional group at the enzyme substrate complex.

The following consideration indicates that one of the two ionizable groups, which show pK values of about 5.8 in the $p\tilde{K}_m{}^{H+}$–pH plot, belongs to the substrate molecule:

On account of the dissociation constant of free ATP[18]

$$\frac{[H]\,[ATP]}{[HATP]} = 3.0 \cdot 10^{-7}\,M \tag{1}$$

the $p\tilde{K}_m{}^{H^+}$–pH curve should show an inflection at pH 6.5, which, however, is not detectable. Therefore, free ATP cannot be the substrate of the Ca^{2+}-dependent ATPase of human erythrocyte membranes. If MgATP is the substrate, the following equilibrium has to be considered in addition:

$$\frac{[Mg]\,[ATP]}{[MgATP]} = 2.15 \cdot 10^{-4}\,M \text{ (ref. 19)} \tag{2}$$

Combining Eqns 1 and 2 leads to

$$[H] = 1.4 \cdot 10^{-3}[Mg]\frac{[HATP]}{[MgATP]} \tag{3}$$

If we assume that HATP and HMgATP do not act as substrates, the H^+ concentration is equal to the apparent dissociation constant, when the ratio

$$\frac{[HATP] + [HMgATP]}{[MgATP]} = 1 \tag{4}$$

i.e. when half of the substrate concentration is inactivated by forming HATP and HMgATP.
The term [HMgATP] of Eqn 4 can be substituted by

$$[HMgATP] = \frac{[Mg]}{5.8 \cdot 10^{-3}}\,[HATP] \text{ (ref. 18)} \tag{5}$$

this yields

$$\frac{[HATP]}{[MgATP]} = \frac{1}{1 + [Mg]\ /5.8 \cdot 10^{-3}} \tag{6}$$

Using Eqn 6 and $[Mg] = 2 \cdot 10^{-3}$ M, Eqn 3 can be solved resulting

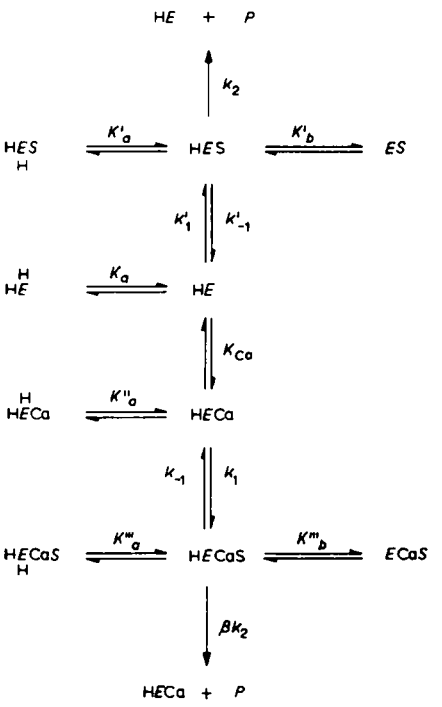
$$[H] = 2.1 \cdot 10^{-6}\,M$$

This value corresponds to pH 5 7, which is in good agreement with the experimental result (*cf* Fig. 5).

Thus, one of the functional groups, which show pK values of about 5.8 in the $p\tilde{K}_m{}^{H^+}$–pH plot, might be due to an ionizable group of the substrate molecule. Furtheron, the derivation justifies the interpretation that MgATP is the substrate of the Ca^{2+}-dependent ATPase of human erythrocyte membranes and that HATP and HMgATP do not act as substrates.

Derivation of the reaction rate equation

The dissociation steps of the free enzyme and the enzyme–substrate complex which appear in Fig. 5, lead to the reaction scheme given below.



The symbols used in this paper are essentially in accordance with the nomenclature used previously[20]. They are defined as follows:

E = free enzyme
$[E]_t$ = total enzyme concentration
S = substrate
P = reaction products
k = reaction rate constant
K = equilibrium constant
$(k'_{-1} + k_2)/k'_1 = K'_m$ = Michaelis constant of the reaction not activated by Ca^{2+} ions
$(k_{-1} + \beta k_2)/k_1 = K_m$ = Michaelis constant of the Ca^{2+}-activated reaction
$\tilde{K}_m^{H+}$ = $[H^+]$-dependent apparent Michaelis constant of the Ca^{2+}-activated reaction
$k_{-Ca}/k_{Ca} = K_{Ca}$ = equilibrium constant of the reaction
$HE + Ca \leftrightharpoons HECa$
$k_{-a}/k_a = K_a$ = equilibrium constant of the reaction
$HE + H \overset{H}{\leftrightharpoons} HE$
$k'_{-a}/k'_a = K'_a$ = equilibrium constant of the reaction
$HES + H \overset{H}{\leftrightharpoons} HES$
$k'_{-b}/k'_b = K'_b$ = equilibrium constant of the reaction
$ES + H \overset{H}{\leftrightharpoons} HES$
$k''_{-a}/k''_a = K''_a$ = equilibrium constant of the reaction
$HECa + H \overset{H}{\leftrightharpoons} HECa$
$k'''_{-a}/k'''_a = K'''_a$ = equilibrium constant of the reaction
$HECaS + H \overset{H}{\leftrightharpoons} HECaS$
$k'''_{-b}/k'''_b = K'''_b$ = equilibrium constant of the reaction
$ECaS + H \overset{H}{\leftrightharpoons} HECaS$

β = charaterizes ratio of rate constants of the reactions $HECaS \rightarrow HECa + P$ and $HES \rightarrow HE + P$ (*cf.* Webb[21])

v = reaction rate in the presence of any substrate and Ca^{2+} concentrations

$V = k_2 [E]_t$ = limiting value of reaction rate at infinite substrate concentration in absence of Ca^{2+}

$\beta V = \beta k_2 [E]_t$ = limiting value of reaction rate at infinite substrate and Ca^{2+} concentrations

$\beta \tilde{V}^{H^+}$ = $[H^+]$-dependent apparent value of βV

In this scheme the different symbols:

$$\overset{H}{HE}, \quad \overset{H}{HECa} \quad \text{and} \quad \overset{H}{HES}, \quad \overset{H}{HECaS}$$

express that the H^+ inhibiting the substrate binding are not identical with those inhibiting the cleavage of substrate. In addition, the dissociation of the substrate is not shown in this scheme in order to facilitate the discussion of this reaction scheme and the equations derived from it.

From this reaction scheme the following reaction rate equation results under equilibrium conditions:

$$v = \frac{V\frac{[S]}{K'_m} + \beta V \frac{[S][Ca]}{K_m K_{Ca}}}{1 + \frac{[H]}{K_a} + \frac{[S]}{K'_m}\left(1 + \frac{[H]}{K'_a} + \frac{K'_b}{[H]}\right) + \frac{[Ca]}{K_{Ca}}\left(1 + \frac{[H]}{K''_a}\right) + \frac{[Ca]}{K_{Ca}}\frac{[S]}{K_m}\left(1 + \frac{[H]}{K'''_a} + \frac{K'''_b}{[H]}\right)} \tag{7}$$

The appearance of straight lines in the $1/v$–$1/[Ca^{2+}]$ plots indicates that

$$V\frac{[S]}{K'_m} \ll \beta V \frac{[S]}{K_m}\frac{[Ca]}{K_{Ca}} \tag{8}$$

Thus, the resulting equation written in the reciprocal form is

$$\frac{1}{v} = \frac{1}{\beta V}\left(1 + \frac{[H]}{K'''_a} + \frac{K'''_b}{[H]}\right) + \frac{K_m}{[S]}\left(1 + \frac{[H]}{K''_a}\right) + \underbrace{\frac{K_a}{\beta V}\left[\frac{K_m}{K'_m}\left(1 + \frac{[H]}{K'_a} + \frac{K'_b}{[H]}\right) + \frac{K_m}{[S]}\left(1 + \frac{[H]}{K_a}\right)\right]}_{x}\frac{1}{[Ca]} \tag{9}$$

The term signed with x is equal to the slope of the straight lines in the $1/v$–$1/[Ca^{2+}]$ plots. At high H^+ concentrations, this term has to be a function of the substrate concentration, especially at low substrate concentrations. As shown in Fig. 1, this

is in agreement with the experimental results. At low H^+ concentrations, however, the increase of the term $K'_b/[H^+]$ leads to

$$\frac{K_m}{K'_m}\left(1+\frac{[H]}{K'_a}+\frac{K'_b}{[H]}\right) \gg \frac{K_m}{[S]}\left(1+\frac{[H]}{K_a}\right) \tag{10}$$

which means that the slope becomes independent from $[S]$. This is also in agreement with the experimental results (*cf.* Fig. 2).

Since the left hand term of Eqn 10 is referred to the reaction steps

$$\underset{H}{HES} \overset{K'_a}{\rightleftharpoons} HES \overset{K'_b}{\rightleftharpoons} ES$$

of the reaction scheme shown above, the $[S]$-independent slopes of the $1/v$–$1/[Ca^{2+}]$ plot at low H^+ concentrations give a strong evidence for the existence of a stable Ca^{2+}-free enzyme–substrate complex. However, this complex must have only very little or even no activity, since the $1/v$–$1/[Ca^{2+}]$ plots are linear at any H^+ concentration. Thus, in the absence of Ca^{2+}, the enzyme is able to bind but not to split the substrate.

In order to get information about the stability of the inactive enzyme-substrate complex, the dissociation constant is evaluated by the following method:

The first derivative of Eqn 9 is converted to

$$\frac{d(1/v)}{d(1/[Ca^{2+}])}\beta V = \frac{K_{Ca}K_m}{K'_m}\left(1+\frac{[H]}{K'_a}+\frac{K'_b}{[H]}\right)+K_{Ca}K_m\left(1+\frac{[H]}{K_a}\right)\frac{1}{[S]} \tag{11}$$

The plot of the left hand side *versus* the reciprocal substrate concentration yields a straight line with a slope of

$$K_{Ca}K_m\left(1+\frac{[H]}{K_a}\right) \tag{12}$$

and with an intercept of

$$\frac{K_{Ca}K_m}{K'_m}\left(1+\frac{[H]}{K'_a}+\frac{K'_b}{[H]}\right) \tag{13}$$

Supposing that the values of K_a, K'_a and K'_b do not differ very much from the values of K''_a, K'''_a and K'''_b, respectively, which can be determined experimentally, at pH 7.0 we get to the following terms:

$$1+\frac{[H]}{K_a}=1.06 \tag{14}$$

and

$$1+\frac{[H]}{K'_a}+\frac{K'_b}{[H]}=1.12 \tag{15}$$

The assumption made above seems to be allowed, since the shift of pK values as the result of Ca^{2+} binding up to 0.5 pH unit causes a deviation of the values estimated below by the factor 1.2 at most.

Using the values of Eqns 14 and 15 and $K_m = 50\ \mu M$ (Fig. 5) then $K'_m = 43\ \mu M$ and $K_{Ca} = 0.92\ \mu M$ are obtained from Fig. 6.

These results indicate that the inactive enzyme–substrate complex is as stable as the active enzyme–Ca^{2+}–substrate complex, *i.e.* the binding of substrate is independent from the presence of Ca^{2+} in the enzyme molecule.

At present it cannot be excluded that Mg^{2+} competes with Ca^{2+} for the Ca^{2+}-binding site. In this case, the constant K_{Ca} would be "apparent" and would have to be corrected for the ratio $[Mg^{2+}]/K_{Mg}$, K_{Mg} being the dissociation constant of the nonactivated E–Mg^{2+} complex.

The active centre of the Ca^{2+}-dependent ATPase

According to the results of Fig. 5 the following groups are present in the active centre of the Ca^{2+}-dependent ATPase of human erythrocyte membranes: (1) One ionizable group with pK of about 5.8, probably an imidazol-nitrogen of histidine[22]. This group is active when dissociated. It is involved only in the process of substrate binding and therefore has to be designated as a "specificity residue" following the nomenclature of Koshland and Neet[23]. (2) A second ionizable group with pK of about 5.8. It may also be an imidazol-nitrogen of histidine[22] and it is also active when dissociated. This group is involved only in the process of substrate splitting. Thus, it has to be designated as a "catalytic residue"[23]. (3) An ionizable group with pK 8.2. This group, possibly an α-amino group of any amino acid[22], is active, when undissociated. It is also involved only in the process of substrate splitting (catalytic residue).

On the basis of these results a model of the active centre of the Ca^{2+}-dependent ATPase is proposed as follows: The model contains the three ionizable groups discussed above. In Fig. 7, they are shown in the state of ionization, in which they are present within pH 5.8 and 8.2 and in which they are active. In addition, the existence of four negative groups (carboxyl groups) is supposed, two of them being used to form the enzyme–substrate complex, while the other two have to bind the Ca^{2+}.

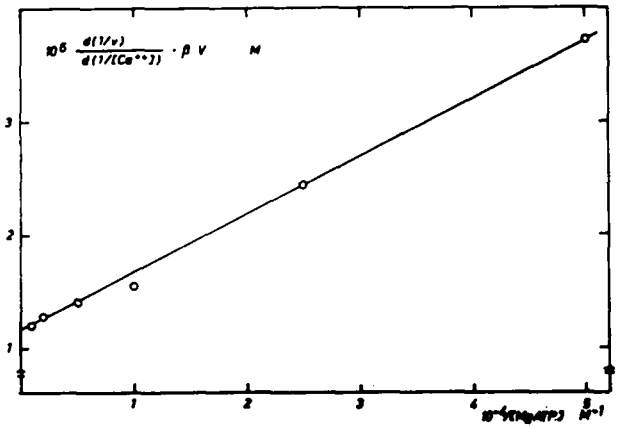


Fig. 6. Plot of $(d(1/v)/d(1/[Ca^{2+}]) \cdot \beta V$ *versus* the reciprocal substrate concentration according to Eqn 11 for the evaluation of K_m and K_{Ca} at pH 7.0. The values of $d(1/v)/d(1/[Ca^{2+}])$ are obtained from Fig. 1, βV from Fig. 5.

The binding of the substrate, which is possible in the absence of Ca^{2+}, probably occurs in the way shown in Fig. 8. Mg^{2+} as the central ion of a hexadental complex is fixed to the enzyme by the presumptive imidazole nitrogen (pK 5.8) and two

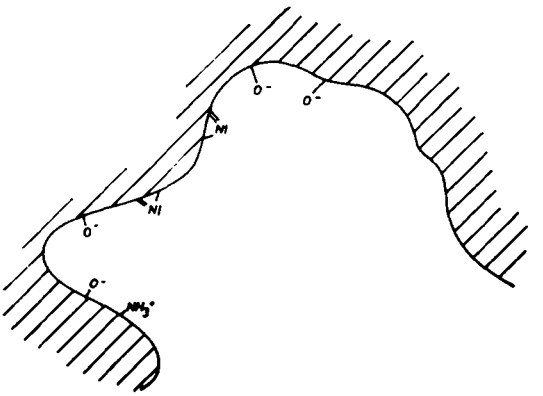


Fig. 7. Tentative model of the active centre of the erythrocyte membrane Ca^{2+}-ATPase. This model contains the three ionizable groups established by kinetic investigations of the pH-dependence of the Ca^{2+} activation. The groups are shown in the state of ionization in which they are present within the range pH 5.8–8.2, and in which they are active. In addition, the existence of four negative groups (carboxyl residues) is assumed. Two of them have to fix the substrate MgATP, while the other two have to bind the Ca^{2+}.

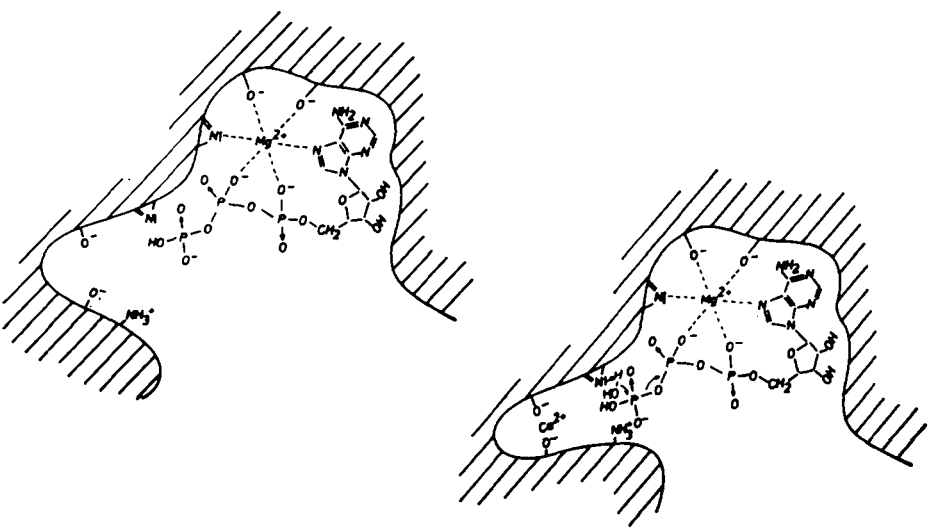


Fig. 8. Binding of the substrate MgATP to the active centre. Mg^{2+} is the central ion of a hexadental complex formed by an imidazole-nitrogen (pK 5.8) and two anionic groups of the enzyme and by the α- and β-phosphate groups and an adenine nitrogen[24,25] of the substrate. In contrast to the drawing the configuration of this complex is a tetragonal bipyramid.

Fig. 9. Cleavage of the substrate requires the binding of Ca^{2+} to a specific site of the active centre, the autosteric site[23]. The binding of Ca^{2+} induces a conformational change with a shift of the catalytic residues towards the γ-phosphate group of the ATP molecule.

anionic groups. The ATP is bound to the Mg^{2+} ion by means of the α- and β-phosphates and of a nitrogen atom of the adenine nucleus[24,25]. This complex is rather stable, as shown by the comparatively low dissociation constant of the enzyme–substrate complex (K_m = 43 μM). In this complex, the substrate cannot be split on account of the great distance between the γ-phosphate group and the catalytic residues, $\geqslant$N| (pK 5.8) and $-NH_3^+$ (pK 8.2). Cleavage of the substrate requires that a Ca^{2+} is bound to a specific site in the catalytic centre. The resulting conformational change shifts the catalytic residues towards the γ-phosphate of the ATP, after which the substrate can be split (Fig. 9). Thus, the Ca^{2+} acts as an autosteric effector, the specific Ca^{2+} binding site being an autosteric site[23] of the enzyme active centre.

In addition an alternative model has to be mentioned, in which the substrate is split in the absence of Ca^{2+} forming a phosphorylated intermediate, which cannot be hydrolyzed without Ca^{2+}. However, the existence of an intermediate, well established in the case of the (Na^+, K^+)-ATPase[26], has not yet been proved in the case of the Ca^{2+}-dependent ATPase of erythrocyte membranes. Investigations are in progress concerning this question.

APPENDIX

Low concentrations of divalent metal ions can be maintained constant by using a buffer, which consists of a strong chelator and the respective metal ion. However, chelators such as nitrilotriacetate, ethylenediaminetetraacetate *etc.*, which may be used in these buffer systems, are protonized to a varying extent in the range of pH 5–10. Thus the metal ion concentration "adjusted" by the buffer system, becomes strongly dependent on the pH value just in that pH range, which is most interesting for enzymatic investigations.

In a buffer system consisting of Ca^{2+} and EDTA ($= L$) the protonization is restricted by the addition of Mg^{2+}, if the sum of the divalent ion concentration is higher than the EDTA concentration.

An equation, which described the dependence of the total Ca^{2+} concentration required to adjust a given concentration of free [Ca^{2+}], from $[L]_t$ and [Mg^{2+}], is derived as follows:

The total concentration of L is given by

$$[L]_t = [L] + [LH] + [LH_2] + [LH_3] + [LH_4] + [MgL] + [CaL] \quad (1)$$

with

$$\frac{[H]\,[L]}{[LH]} = K_1 \quad (2)$$

$$\frac{[H]\,[LH]}{[LH_2]} = K_2 \quad (3)$$

$$\frac{[H]\,[LH_2]}{[LH_3]} = K_3 \quad (4)$$

and

$$\frac{[H]\,[LH_3]}{[LH_4]} = K_4 \tag{5}$$

Eqn 1 is rewritten as

$$[L]_t = [L]\left(1 + \frac{[H]}{K_1} + \frac{[H]^2}{K_1K_2} + \frac{[H]^3}{K_1K_2K_3} + \frac{[H]^4}{K_1K_2K_3K_4}\right) + [MgL] + [CaL] \tag{6}$$

The concentration ratio [free divalent metal ion]/[complex] is expressed as

$$\frac{[Mg]\,[L]}{[MgL]} = K_{MgL} \tag{7}$$

and

$$\frac{[Ca]\,[L]}{[CaL]} = K_{CaL} \tag{8}$$

Combination of these two equations yields

$$[MgL] = [CaL]\frac{[Mg]}{[Ca]}\frac{K_{CaL}}{K_{MgL}} \tag{9}$$

Insertion of Eqns 7 and 9 into 6 allows solution for [CaL]. With

$$[Ca]_t = [CaL] + [Ca] \tag{10}$$

we find

$$[Ca]_t = \frac{[L]_t}{\frac{K_{CaL}}{[Ca]}\left(1 + \frac{[H]}{K_1} + \frac{[H]^2}{K_1K_2} + \frac{[H]^3}{K_1K_2K_3} + \frac{[H]^4}{K_1K_2K_3K_4}\right) + 1 + \frac{[Mg]}{[Ca]}\frac{K_{CaL}}{K_{MgL}}} + [Ca] \tag{11}$$

The total concentration of Mg^{2+}, which has to be present to maintain the desired concentrations of free Ca^{2+} and free Mg^{2+}, can be calculated easily by Eqn 9 and

$$[Mg]_t = [MgL] + [Mg] \tag{12}$$

It should be emphasized that the Ca^{2+} level, adjusted by a buffer of this type, is constant within a pH range of about 6–10. For further information see ref. 27.

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